Phosphate Nutrition of *Rhizobium spp*.

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This study was conducted to determine the behavior of 40 strains from six species of *Rhizobium* in liquid defined media containing orthophosphate at levels likely to be encountered naturally, ranging from the high concentrations expected in nodules and artificial media to the low concentrations of soil solutions. Storage capacity in strains with high levels (2 mM) of P and ability to utilize this stored P for growth after transfer to low levels (0.06 μ M) of P varied with each strain. Storage varied from about 1 to 2% P (dry weight) for all strains, with the number of generations supported dependent on the quantity of P stored and on the utilization efficiency. The ability to store P at high levels is probably less important than the uptake and utilization efficiency of P supplied at low levels. Strains varied greatly in tolerance to low levels of P maintained in solution by an iron oxide buffering system. Differences in growth rate at low levels of P were large enough to be agronomically important.

Successful rhizobia must be able to spread and persist in soils and rapidly colonize the host root (8) under extremely diverse P environments. The P gradient from rhizosphere to nodule may be as much as five orders of magnitude (1). Nodule tissue, which may contain concentrations of 10^{-4} to 10^{-2} M dissolved Pi (6), probably provides the same luxury P conditions as conventional laboratory media. Soil solutions commonly range from 10^{-5} to 10^{-7} M P in solution (9), and rhizospheres may reach concentrations of 10^{-8} M P (7). To deal with this variation, a P-efficient strain has three mechanisms: (i) ability to store large quantities of P, (ii) utilization efficiency of internal P, and (iii) uptake efficiency at low external concentrations. Mechanisms (i) and (ii) would be most important when rhizobia move from nodules or artificial media into soil. Mechanism (iii) would be most important for long-term growth and persistence in the soil.

Recent work on the performance of *Rhizobium japonicum* under various phosphate regimes has shown considerable strainto-strain differences in growth at low levels of P (2, 3). The purpose of this work was to determine the scope of strain variability in response to P stress.

MATERIALS AND METHODS

Strain designations, hosts, and sources are listed in Table 1. All 40 strains were maintained under refrigeration on slants of nutrient medium (see below) with yeast-agar-2,000 μ M P.

The liquid nutrient media contained 1.1 g of sodium glutamate, 3 g of galactose, and 3 g of arabinose per liter of distilled water, with inorganic nutrients at the following micromolar concentrations: MgS0₄, 300; FeEDTA, 50; MnS0₄, 2; ZnS0₄, 1; CUS0₄, 0.5; Na₂Mo0₄, 0.1; CoC1₂, 0.02. Calcium was supplied in the form of CaCl₂ at 300 μ M, except in cultures of *Rhizobium meliloti*, which were supplied with 1,500 μ M Ca because of their high calcium requirement under conditions of low levels of P (6; D. P. Beck and D. N. Munns, manuscript in preparation). Vitamins were supplied as follows: biotin, 0.1 ppm (1.0 μ g/ml); thiamine, 1.0 ppm; and pantothenic acid, 1.0 ppm. The pH was adjusted to 5.5 with HCl before autoclaving. To main

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tain buffered concentrations of phosphate at low levels in solution, we used an iron oxide dialysis system (3). The concentration of P in solution depended on the amount added with the iron oxide. The oxide was powdered limonite (Ward's Natural Science Est., Rochester, N.Y.) consisting primarily of goethite (aFe00H). This was phosphated in 150-g lots by shaking for 6 days in 1.5 liters of $CaCl_2$ (0.01 µM) with the appropriate amount of KH_2PO_4 (3), followed by filtration and air drying. Each culture received a slurry containing 3 g of oxide and 4 ml of distilled water contained in a section of dialysis tubing ("Spectrapor," molecular weight cutoff, 12,000 to 14,000; Spectrum Medical Industries, Los Angeles, Calif.), which was knotted at both ends and added to 37 ml of culture medium in a 125-ml Erlenmeyer flask. Cultures were autoclaved for 30 min and left for 3 days at 25 to 27°C to equilibrate. The desorption isotherm (3) was determined at the end of this time by analysis of the liquid medium. The luxury P control contained KH₂PO₄-K₂HPO₄ at a concentration of 2,000 µM. The low P treatment, with no P added, and the oxide cultures received $250 \ \mu M \ K_2 SO_4$ to ensure an adequate supply of potassium.

Phosphate concentrations were determined by a phosphomolybdate blue procedure (10). Mannitol suppressed color development, whereas arabinose-galactose apparently caused no interference.

Three sets of experiments were carried out.

(i) The amount of phosphorus stored was measured at a luxury level of P (2,000 μ M) and a high level of P representing that found in fertile soil (5.0 μ M). The 5 μ M level was maintained by the oxide dialysis system, expanded in proportion to 16 g of oxide per 200 ml of medium in 500-ml Erlenmeyer flasks. At each level of P, two replicates were grown to turbidity (10⁷ to 10⁸ cells per ml) and then centrifuged at relative centrifugal force 10,000 for 20 min, suspended in 0.01 μ M CaC1₂, and recentrifuged. The pellet was dried for 20 to 24 h at 60°C, weighed, then analyzed after Kjeldahl digestion for P.

(ii) Growth curves after P accumulation were plotted in which growth was monitored after transfer from medium with luxury levels of P (2,000 μ M) to low levels of P (0.06 μ M). The inocula, grown to turbidity, were diluted 1:50 in medium with low levels of P, and 0.1 ml of dilution was added to 37 ml of media to give an initial density of 10³ to 10⁵ cells per ml. Cell growth was followed in "runout" culture at

 TABLE 1. Strain designations, hosts, and sources of 40 strains of Rhizobium screened

Host plant	Strain no. and source
Arachis hypogea	8A16 ^{<i>a</i>} ; TAL1000 ^{<i>b</i>} and TAL1371 ^{<i>b</i>}
Cicer arietinum	TAL480 ^b and TAL1148 ^b ; NA936 ^c
Centrosema spp	$TAL651^{b}$ and TAL1146 ^b
Glycine max	USDA 6 ^d , USDA 110 ^d , USDA 138 ^d ,
-	and USDA 142^d
Lens culinaris	TAL638 ^b and TAL640 ^b
Leucaena leucocephala.	TAL582 ^b and TAL1145 ^b
Medicago sativa	
5	USDA 1029^d and USDA 1031^d
Phaseolus lunatus	
Phaseolus vulgaris	$CIAT45^{e}$ and CIAT904 ^e · USDA 2668 ^d
Pisum sp., Vicia sp., etc.	$-175G10^{f}$, 128C53 ^f , 92F1 ^f , 92F2 ^f
Trifolium spp.	162×46^{a} 162×97^{a} and 162×99^{a}
Viena radiata and Viena	1102 · · · 10 ; 102 · · · / ; und 102 · · · //
unguiculata	TAL 209 ^b TAL 309 (CB756) ^b
	$TAI 420^{b} TAI 441^{b} TAI 169^{b} and$
	TAL 310^{b} ; BR404 ^g and BR418 ^g

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intervals for 7 days by viable cell drop counts on yeast-arabinosegalactose plates (11). At each time, six replicate, $41-\mu 1$ drops were counted from each triplicate culture. Analysis of variance was carried out on log₁₀ transforms of the counts.

(iii) Screening for growth at low levels of P was carried out at concentrations most likely to be encountered by bacteria in the soil, with the high level representing the solution concentration in a P-fertile soil (5.0 µM), the medium level representing a Pdeficient soil (0.5 µM), and the low level representing a Pdepleted rhizosphere (0.06 μ M). Inocula for the experiments were grown in P medium with low levels of P to eliminate storage effects and diluted to 10^3 to 10^4 cells per ml at the beginning of the experiment. Selected strains were chosen for variability of performance in the earlier P-runout experiments, and their growth was monitored by drop counts at intervals for 7 days after inoculation into media at three buffered levels of P, plus a 2,000 μ M luxury level of P control. The least significant difference (P = (0.05) was calculated from the analysis of variance on \log_{10} transforms of the counts. The culture medium was analyzed at intervals for levels of P to measure the buffering performance of the oxide, and pH was measured near the end of each experiment to evaluate the ability of the strain to modify the pH of the culture medium.

RESULTS

Phosphate storage of the strains screened varied considerably, ranging from about 1 to 2% of dry weight in 2 mM P and 0.25 to 1.7% in cells grown at 5 μ M P (Tables 2 and 3). In general, slow growers stored a greater quantity of P than did the fast growers. Strains also differed in their ability to grow on stored P after transfer into medium with low levels of P (Fig. 1). The number of generations produced in runout on

 TABLE 2. Phosphorus accumulation at two solution levels of P

 and generations supported after transfer into low levels of P (fast-growing strains)

Rhizobium species	<i>Rhizobium</i> strain	% Dry wt with the following P content:		No. of genera- tions	Starting population (log
		2 mM	5 µM	supported ^a	cells/ml)
R. trifolii	162 × 99	0.95	0.28	3.5	5.0
	162 × 46	1.25	0.34	4.8	
R. legumi-	92F1	1.10	0.44	5.75	4.3
nosarum	175G10	1.35	0.60	7.1	
R. phaseoli	CIAT45	1.20	0.30	7.6	5.5
•	CIAT904	0.95	0.44	0.7	
	USDA 2668	1.00	0.26	5.6	
R. meliloti ^b	USDA 1021a	1.80	0.56	6.6	5.0
	102F28	1.40	0.43	3.0	010

^a After transfer from 2 mM P into 0.06 µM P.

^b R. meliloti grown at high Ca^{2+} (1.5 mM); others grown at 0.3 mM Ca.

stored P varied greatly and evidently depended not only on the amount of P stored and on utilization efficiency, but also on the inoculum size.

The inoculum size would have no effect on the number of generations if the inoculant itself were the only source of P, but real media contain P impurity at some fixed low level which becomes a more significant part of the total with a smaller inoculum size. We used small inocula to ensure that P, and not something else, remained limiting at the cessation of growth. Our inoculum size varied because it is undesirable to hold inocula several days while waiting for viable counts. The P contained in the inoculum decreased from about 10^{-6} to about 10^{-8} g per culture as the inoculum decreased from 10^5 to 10^3 cells per ml of culture,. Phosphate present as an impurity in the medium with low levels of P (0.06 μ M) amounted to 8 x 10^{-8} g per culture. This was less than 10% of the total P when the inoculum size was 10^5 cells per ml, but it was 90% of the total

TABLE 3.	Phosphorus ac	cumulation at	t two soluti	on levels of P
and generation	ons supported	after transfer	into low P	(slow-growing

		strains			
Host	<i>Rhizobium</i> strain	% Dry wt with the following P content:		No. of genera- tions	Starting population (log
		2 mM	5 μΜ	supported ^a	cells/ml)
Arachis spp.	TAL1371	1.60	0.75	4.65	5.6
	8A16	1.40	0.48	4.5	
Cicer arie- tinum	TAL480	1.10	0.42	3.65	4.3
	TAL1148	1.45	1.20	7.0	
Vigna radiata	TAL209	1.05	0.58	2.6	4.3
	TAL441	1.45	0.86	4.9	
Vigna ungui-	TAL169	1.65	1.12	4.7	6.0
culata	TAL309	1.80	1.20	5.0	
G. max	USDA 142	1.6	1.30	1.8	4.5
	USDA 110	2.1	1.70	3.5	

^a After transfer from 2 mM P into 0.06 µM P.



FIG. 1. Growth after transfer from medium with luxury levels of P (2 mM) to medium with low levels of P (0.06 μ M) in runout experiments. *R. meliloti* N102F28 was grown with 1.5 mM Ca; the others were grown with 0.3 mM Ca.

when the inoculum was only 10^3 cells per ml. The decrease in inoculum size decreases the final count achieved, because there is less P; but it allows more generations to be produced because the fixed amount of P impurity goes further.

Data for Tables 2 and 3 were selected for the similarity of inoculumn size to permit valid, within-host comparisons of the number of generations supported. The number of generations supported corresponded with the quantity of P stored. Values between hosts cannot be compared because inoculum levels varied.



FIG. 2. Frequency diagram of P utilization efficiency in 40 strains. Phosphate utilization efficiency is expressed as number of viable cells produced per microgram of P available (from inoculant cells and from medium with low levels of P).

To obtain a measure of P utilization efficiency for comparing all 40 strains screened, we divided the maximum population attained in each runout culture by the total P input, including stored P from the inoculum plus P initially present in the medium with low levels of P. These values, reported in Fig. 2 as cells per gram of P, give an approximation of combined efficiency of use of both stored P and external P at low solution concentration.

In general, there was a wide range of efficiency. The most efficient fast-growing strains were from *Rhizobium phaseoli*, the least efficient were from *R. meliloti*. The representative slow-growing rhizobia, composed of *R. japonicum* and the cowpea miscellany, in general, tended to be more efficient, but they also included the least efficient of all strains screened.

As a group, *R. meliloti* received slightly different treatment than the other strains tested, as they were unable to utilize stored P or low solution concentration of P at the 0.3 mM calcium level that is adequate for the strains of other species. Accordingly, we grew *R. meliloti* at 1.5 mM Ca. At low levels of Ca, no reproduction occurred after transfer of *R. meliloti* to low levels of P.

Although nodule bacteria may be exposed to millimolar concentrations of P (6), concentrations in the soil are unlikely to exceed 5 μ M (9). For most strains, the amount of P stored in 5 μ M P cultures was apparently sufficient for only a few generations, so that these bacteria are unlikely to accumulate enough P when growing in soil to carry them through colonization of the rhizosphere. It is probably important that they be able to take up and utilize P at low levels.

Strains to be screened at low levels of P were chosen according to their efficiency in P runout experiments. Select-



FIG. 3. Growth of two isolates each of R. trifolii and R. leguminosarum at three low-buffered levels of phosphate. LSD, Least significant difference.



FIG. 4. Growth of two R. *meliloti* (alfalfa) isolates at three lowbuffered levels of P, with a high concentration (1.5 mM) of calcium. LSD, Least significant difference.

ed efficient and inefficient strains were grown at low-buffered levels of P.

The dialysis system was able to buffer the phosphate concentration at low levels until the population in the cultures reached 10^6 to 10^7 cells per ml, at which point bacterial uptake exceeded the rate of P movement into solution. Growth curves were plotted only as long as the buffering capacity remained; with slow-growers it remained through 4 days, and with fast-growing strains it remained through 48 to 72 h.

Within each group of fast-growing rhizobia, strains showed little or no difference in growth at low levels of P (Fig. 3, 4, and 5). Growth at high levels of P (5 μ M) was significantly higher for strains of Rhizobium leguminosarum, Rhizobium trifolii, R. phaseoli, and R. meliloti than at low levels of P; but, except for R. meliloti, the differences were not large. The growth of two strains of *R. meliloti* in high levels of calcium is depicted in Fig. 4. No growth was achieved in low levels of calcium at the three low levels of P. Strains of R. meliloti could not reproduce at the lowest level of P, regardless of the calcium concentration. No significant differences in growth existed between the treatments with high and luxury levels of P for any fast-growing strains, except for Leucaena isolate TAL582. In contrast to the other fast-growing strains tested, the two isolated from Leucaena spp. (Fig. 5) showed large significant differences in growth rate at all low P levels after 2 days, although growth was equal in 2 mM P.

Four strains of slow growers showed a larger range of variation in sensitivity of low levels of P (Fig. 6). All grew at similar rates in luxury P, but strains USDA 142 (R. *japonicum*) and TAL420 (cowpea miscellany) grew slowly at the lower P levels. The most efficient strains, USDA 110 and TAL169, produced equivalent populations, irrespective of the P concentration.

All the strains raised the pH of the growth medium to some extent. In general, the magnitude of the shift corresponded to the population increase, with maximum populations raising the pH by one-half to two units.

DISCUSSION

Low levels of phosphate reduced the growth rate of most strains tested and stopped growth in a few cases. The wide

range of P utilization efficiency shown suggests much variation throughout the genus. Colonization of the rhizosphere and nodule initiation are growth rate dependent (4), and growth reduction by low levels of P could be critical. A beneficial effect of P on nodulation in greenhouse and field trials has been shown with several species and is probably not due entirely to increased plant vigor (5, 12).

The buffering system was able to maintain solution concentrations at realistic levels much as soil does. Growth differences at these levels point to the importance of considering the nutritional environment when choosing a suitable strain.

The ability of rhizobia to store P and utilize it for subsequent growth is strain dependent. Storage may be a mechanism for utilizing exposure to high levels of P to assist subsequent colonization of the soil or rhizosphere. Nodule P probably supplies this exposure.

The slow-growing; group, in general, appeared to store P in larger quantities. Polyphosphate granules occur in cells of *R. japonicum* above 1.4% P (cell dry weight) (2) and may provide additional storage above cytoplasmic levels or act as a detoxification mechanism. Some fast-growing strains might also use this form of P storage to a degree. Electron micrographs of *R. trifolii* and *R. meliloti* strains have shown no evidence of granules with 1.0 and 1.4% stored P, respectively (unpublished data), but might do so if P exceeded a threshold internal concentration.

Ability to store P at high levels is probably not as important as efficiency in utilizing what P is available. The high P levels found in our medium with luxury levels of P are likely only in the laboratory, or perhaps, in nodule tissue (6). Most soil solutions contain P at concentrations of less than 1 μ M (9), and the ability to utilize these lower solution levels should affect proficiency in colonization and persistence. This ability varied. Several slow-growing strains and both strains of *R. meliloti* could not grow at the lowest P level,



FIG. 5. Growth of two isolates each of R. phaseoli and Leucaence rhizobia at three low-buffered concentrations of phosphate, with data for luxury levels of P (2 mM) for Leucaena sp. LSD, Leas significant difference.



FIG. 6. Growth of four slow-growing strains at low-buffered levels of phosphate, with 2 mM luxury level of P control for R. *japonicum* strains. LSD, Least significant difference.

which corresponds to levels expected to develop in rhizospheres in P-deficient soils.

The screening procedure with low levels of P may be a useful method for detecting tolerant strains. One or two counts at a selected low level might be adequate. More convenient would be an agar slant or plate screening method, but even purified agar provides about 2 μ M endogenous P in solution which is too high for effective screening.

Tolerance of rhizobia to low P, as determined in the laboratory, corresponded to the ability to nodulate *Glycine max*, *Vigna radiata*, and *Phaseolus vulgaris* in greenhouse

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experiments in P-deficient soil (unpublished data). Differences in nodulation, plant dry weight, and plant N content indicate that strain variability in tolerance to low levels of P may be agronomically important.

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